AD-A183 646 PROCESSING OF ARCHAEBACTERIAL INTRON-CONTAINING TRNA GENE TRANSCRIPTS(U) OHIO STATE UNIV COLUMBUS DEPT OF MICROBIOLOGY C J DANIELS 31 JUL 87 N00014-86-K-0645 1/1

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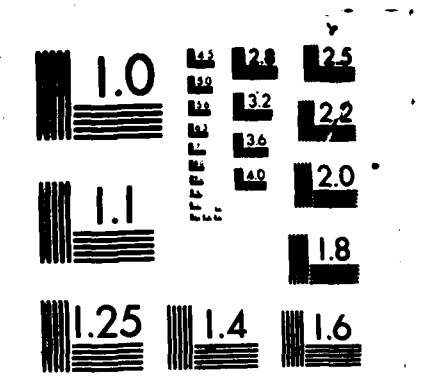

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ORT DOCUMENTATION PAGE

1a. (U)	1b. RESTRICTIVE MARKINGS NA
2a. SECURITY CLASSIFICATION AUTHORITY NA	3. DISTRIBUTION / AVAILABILITY OF REPORT Distribution Unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE NA	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) The Ohio State University	5. MONITORING ORGANIZATION REPORT NUMBER(S) NA
6a. NAME OF PERFORMING ORGANIZATION The Ohio State University	6b. OFFICE SYMBOL (If applicable) NA
6c. ADDRESS (City, State, and ZIP Code) Dept. of Microbiolodgy, O.S.U. 484 W. 12th Ave. Columbus, OH 43210	7a. NAME OF MONITORING ORGANIZATION Office of Naval Research
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Office of Naval Research	8b. OFFICE SYMBOL (If applicable) ONR
8c. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000	7b. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N0014-86-K-0645
	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61153N	RR04106		

DTIC ELECTE AUG 11 1987 S D

11. TITLE (Include Security Classification)

Processing of Archaebacterial Intron-Containing tRNA Gene Transcripts

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 7/86 TO 7/87	87-7-31	9

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Archaebacteria, Intron, RNA splicing, RNAase P Halobacteria, tRNA, DNA sequencing
08			

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

An assay for the Halobacterium volcanii $tRNA^{Trp}$ intron endonuclease has been developed. This enzyme accurately cleaves the H. volcanii intron containing $tRNA^{Trp}$ substrates, but does not act on a typical eukaryotic intron-containing precursor, yeast $pretRNA^{Phe}$. Substrate deletion analysis indicates that "mature" tRNA structures and intron sequences are not absolutely required for recognition and processing by this enzyme. These findings suggest a sequence specific recognition pattern. An activity capable of removing 5' flanking sequences (RNAase P) from intron and non-intron containing pretRNAs has also been partially purified from this organism and techniques have been developed to investigate this activity in Thermoplasma. We are also investigating the overall distribution of introns in H. volcanii tRNA genes. Using the technique of orthogonal field gel electrophoresis two large BamHl restriction fragments which contain the majority of tRNA genes in this organism have been identified.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	(U)

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
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DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

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1. Project Goals:

A. To determine the mechanism of tRNA intron processing in the halophilic archaebacteria.

B. Characterize and compare the enzyme(s) responsible for the removal of 5'-flanking sequences from halophilic and sulfur-dependent tRNA gene transcripts.

C. Examine the structure and distribution of tRNA introns in the halophilic archaebacteria.

2. Accomplishments:

A. Intron processing mechanism

We have succeeded in our primary objective of determining the nature of the reaction occurring with in vitro synthesized intron containing $tRNA^{Trp}$ precursor and extracts from Halobacterium volcanii. The initial observation that both endonuclease and ligase activities were present in crude extracts has been verified. However, the majority of activity observed in the extract was due to endonuclease; only 2-5% of the cleaved products were religated to give "mature" tRNAs. We have analyzed the endonuclease reaction in some detail.

The endonuclease has been partially purified from H. volcanii by polyethylene glycol precipitation of high speed supernants followed by chromatography on DEAE Sephacel and hydroxylapatite. This scheme has led to an approximate 100 fold purification and removes all RNAase P and other ribonuclease activities (see below). Using a combination of direct RNA sequence analysis and the analysis of terminal oligonucleotides, it was established that this endonuclease accurately cleaved the in vitro generated substrate at the expected processing sites (Figure 1). An analysis of the biochemical requirements of the reaction indicated that this enzyme required 20mM Mg^{2+} ions or 1mM spermidine for maximum activity. In contrast to its predicted "natural" environment, the enzyme did not require monovalent ions. The enzyme was inhibited by KCl concentrations greater than 50mM in the standard assay. Attempts to purify activity from extracts prepared in high salt (2M KCl) and assaying under high salt conditions have not been successful.

An important part of this analysis is to determine the relationship of the H. volcanii tRNA intron endonuclease to its eukaryotic counterpart. There are two aspects of this relationship which we are examining, substrate recognition and the chemistry of the reaction. The eukaryotic endonuclease is capable of cleaving a number of precursors all having different primary sequences but similar "mature" tRNA structures. It is this observation and that the eukaryotic enzyme is inhibited by mature tRNA which suggested that the enzyme recognizes the "mature" tRNA structures rather than primary or intron sequences. We anticipated that the H. volcanii enzyme would behave similarly. However, when the H. volcanii enzyme was presented with mature tRNA from yeast as a

competitive inhibitor no inhibition was observed (Figure 2). The enzyme was also unable to process the intron-containing yeast $tRNA^{Phe}$ precursor (Figure 3). To further examine the substrate requirements of the enzyme a number of altered substrates were prepared. An analysis of intron and exon deletions indicates the H. volcanii enzyme does not require the ability of the substrate to form "mature" tRNA-like structures. It also does not have an absolute requirement for extended base pairing of the anticodon region since the intron sequences involved in this interaction can also be removed without affecting the endonucleolytic cleavage of the substrate. It would appear that the H. volcanii enzyme is specific for the $tRNA^{Trp}$ precursor RNA, possibly recognizing primary sequence at or near the splice site. If this is true, it would represent a departure from the current proposals that a single enzyme processes all tRNA precursors and may provide some insight into the origin of these enzymes.

The chemistry of the yeast and HeLa cell tRNA intron endonucleases have been examined in some detail. The most revealing property of the reaction is the fate of the phosphate group at the splice junction. In both cases the α phosphate at the exon 1 -intron and intron-exon 2 boundaries are transferred to the 3' end of exon 1 or excised intron in the form of a 2',3' cyclic phosphate leaving 5' hydroxyls on the intron and exon 2. In the yeast mechanism, the 2',3' cyclic phosphate is subsequently opened to a 2' phosphate whereas in the HeLa all reaction the 2',3' phosphate remains intact.

An analysis of the 3' terminal oligonucleotide from the excised $tRNA^{Trp}$ intron and the 5' end of the released exon 2 indicate a transfer of phosphate from the splice site to the 3' terminal nucleotide of the intron. Preliminary analysis suggests that this phosphate is present on the 3' hydroxyl, no 2' or 2',3' cyclic derivatives have been detected. This is consistent with the nuclear tRNA splicing mechanisms but does not provide us with the means to distinguish between the yeast or HeLa mechanisms. We cannot exclude the possibility that a contaminating phosphatase hydrolyzes a 2',3' cyclic phosphate intermediate. We have also been unable to detect a kinase activity in our preparations which could phosphorylate the 5' hydroxyl of the released second exon, which is an intermediate step in the yeast splicing mechanism. The status of the 3' end of the first exon when released by the endonuclease has not been examined in detail. The ability to label the 5' termini of the excised intron by polynucleotide kinase and $\gamma^{32}P$ ATP would strongly suggest that a phosphate transfer has occurred as observed for the intron-exon 2 boundary.

The accuracy and the chemistry of the endonuclease appears to be similar to those of the yeast and HeLa cell enzymes. In contrast, the archaebacterial enzyme appears not to have a requirement of "mature" tRNA structures and may be specific for the $tRNA^{Trp}$ precursor.

Utilizing the products of the endonuclease reaction, exon 1 and exon 2, we have begun to search for a tRNA ligase activity. To date, our analysis has shown that ligation does not occur in the partially purified endonuclease preparation either in the presence or absence of ATP. A

separate purification scheme for ligase, using both low and high salt conditions is being examined.

B. Ribonuclease P activity

Preliminary experiments using the intron containing H. volcanii $tRNA^{Trp}$ indicated that an enzymatic activity capable of removing the 5' leader sequence was present in crude extracts. The specificity of cleavage when compared to other nucleases (S1 and RNAase A) suggested that this activity was analogous to RNAase P. Using the intron-containing $tRNA^{Trp}$ substrate, we devised a purification scheme to separate the intron endonuclease and RNAase P activities. RNAase P was partially purified by polyethylene glycol precipitation, DEAE chromatography and glycerol gradient fractionation. To simplify the assay, and to analyze the activity on a non intron-containing substrate, we have prepared a second substrate. The H. volcanii $tRNA^{Val}$ clone which we have characterized previously was cloned into the T7 expression vector such that it would produce a runoff transcript containing a small 5' leader sequence. When this tRNA precursor was used as substrate two discrete products were generated (Figure 4). The sizes of the products were consistent with a single endonucleolytic cleavage at the 5'-terminus of the mature tRNA. We are currently evaluating the accuracy of the reaction.

The eubacterial RNAase P enzymes that have been characterized are ribonucleoprotein complexes containing a small protein and a RNA of approximately 300 nucleotides. An interesting property of this enzyme is that the RNA is the enzymatic component. Consequently we are investigating the possibility that the H. volcanii enzyme also has an enzymatically active RNA component. In our analysis we have observed several small RNAs associated with the activity. We have not been able to demonstrate a requirement for RNA by pretreatment of the enzyme with micrococcal nuclease since EGTA, which is required to inactivate the enzyme, also inactivates the RNAase P activity. Subsequent to our work, we have learned that Dr. Altman's laboratory at Yale has partially purified this same enzyme and shown a requirement for RNA. We have now begun a collaboration with Dr. Altman to try and complement an E. coli RNAase P temperature sensitive lethal mutant with cloned fragments from H. volcanii, Thermoplasma and Methanothermus fervidus to identify the RNA component. We remain in contact with Dr. Altman's laboratory who has now informed us that a H. volcanii protein can function in a reconstitution assay using E. coli RNAase P RNA. We are continuing our characterization of the RNA components of this activity.

We are also beginning our analysis of Thermoplasma RNAase P activity. Methods have been established in our laboratory to culture Thermoplasma both on a small scale (1-1000 ml) and in the fermentor (20 l). Although the yield of cells (1g/liter) is significantly less than that for H. Volcanii (5g/liter), the availability of a 20 l fermentor will provide sufficient cells for protein purification. Initial assays will be performed with the H. volcanii $tRNA^{Val}$ substrate. We have also cloned the Thermoplasma $tRNA^{Lys}$ gene and have constructed clones in M13 which when cloned into the T7 vector will produce precursor tRNA with a short 5' leader and essentially no 3' flanking sequences.

C. Intron structure and distribution

We had proposed originally to screen for intron-containing tRNA precursor molecules in the population of minor RNAs of H. volcanii by using these RNAs as substrates with the $tRNA^{Trp}$ processing enzyme. Those RNAs which processed would then be used as a probe to clone their respective genes. Using this approach we have screened 13 potential precursors; all but one gave a negative result with the $tRNA^{Trp}$ endonuclease. The single RNA which did response to the enzyme appeared by size characteristics to be the $tRNA^{Trp}$ precursor. In view of our finding that the $tRNA^{Trp}$ endonuclease is specific for $tRNA^{Trp}$ precursor, this approach does not have general applicability we had anticipated. We are now examing an alternative approach which will be less selective. Using purified mature tRNAs and RNA ligase an 18 nucleotide poly A tail can be added to the tRNAs. cDNAs of these RNAs can be synthesized by reverse transcriptase using an oligo dT primer. A Northern analysis of total RNA from H. volcanii using this cDNA as a probe should detect precursor tRNA molecules. While this approach will be limited by the relative amounts of the tRNA species in the original population, it will detect all precursors (both intron-containing and large primary transcripts with no introns such as dimers) and not be subject to the specificity problem associated with the $tRNA^{Trp}$ enzyme. Preliminary experiments with RNA populations greater in size than mature tRNAs prepared by gel purification or HPLC should provide a means to quickly evaluate this technique. Furthermore, the $tRNA^{Trp}$ precursor provides a specific control.

We are also pursuing a more comprehensive analysis of H. volcanii tRNA genes. The focus of this analysis is on isolating large DNA fragments which have been shown to contain many tRNA genes. In collaboration with Dr. Ford Doolittle's laboratory, it has been determined that many of the H. volcanii tRNA genes are located on two large BamHl fragments (Figure 5). Using a field inversion gel electrophoresis apparatus now in our laboratory we are preparing to isolate these fragments. Once isolated, we will prepare subclones for analysis. We are hopeful that this approach will quicken our analysis.

3. Plans for the Next Year:

A. Continue to analyze the H. volcanii $tRNA^{Trp}$ endonuclease. Using our knowledge of substrate specificity we are developing strategies to further purify this enzyme by affinity chromatography.

B. Develop an assay for the $tRNA^{Trp}$ ligase reaction using the endonuclease products as substrate. To accomplish this it maybe necessary to first purify a 2',3' cyclase which could convert the exon 1 3' terminus to a 2',3' cyclic phosphate.

C. Further characterize the RNA component of the H. volcanii RNAase P enzyme. In view of the progress being made by Dr. Altman in identifying the protein component, we will focus on the RNA moiety of the enzyme.

D. Establish a Thermoplasma RNAase P assay system with H. volcanni tRNA genes, in particular those genes localized to the two "tRNA-rich" BamHl fragment.

4. Presentations

A tRNA Intron Endonuclease from the Archaebacteria. Leo D. Thompson and Charles J. Daniels. American Society for Microbiology, March, 1987.

Transfer RNA Processing in the Archaebacteria. Leo D. Thompson and Charles J. Daniels. UCLA Symposia on Molecular and Cellular Biology: "Evolution of Genes and Genomes". March, 1987.

5. Personnel:

Leo D. Thompson is a graduate student supported by this grant. He has carried out most of the work on the $tRNA^{Trp}$ endonuclease. Ann Melick, a parttime technician, and Greg Gorospe, an undergraduate have worked on the RNAase P and genomic cloning projects.

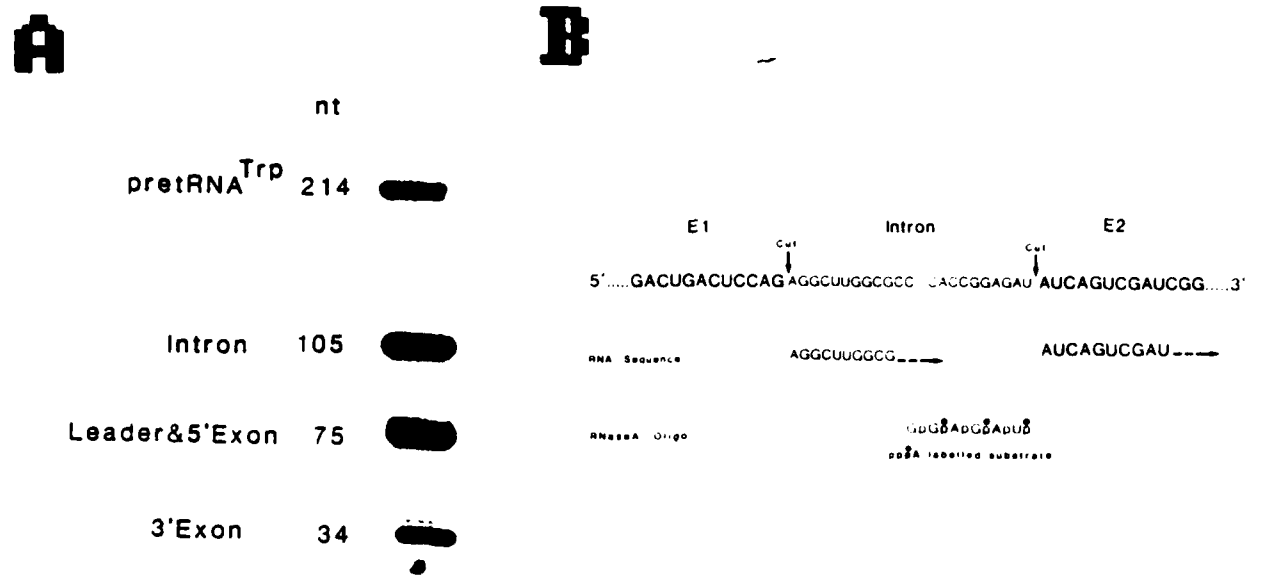


Figure 1. Processing of the intron-containing tRNA^{Trp} precursor by partially purified H. volcanii endonuclease. Panel A, products produced from the 214 nucleotide precursor under standard assay conditions. Panel B, accuracy of the cleavage reaction as determined by direct RNA sequencing and oligonucleodide analysis.

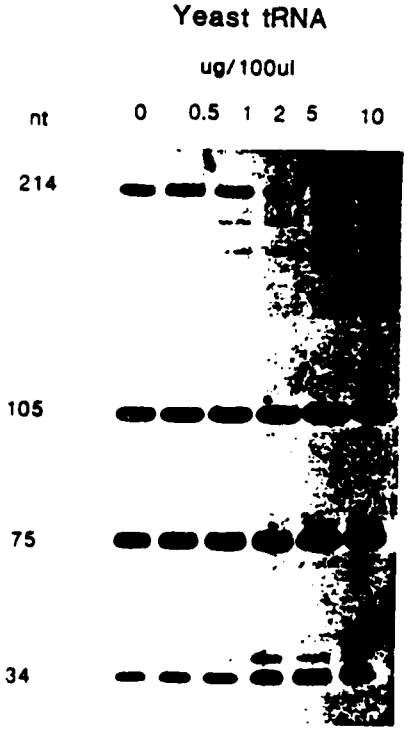


Figure 2. The affects of yeast tRNA on the H. volcanii tRNA^{Trp} endonuclease. Processing of labeled pretRNA^{Trp} was examined in the presence of increasing amounts of unlabeled bulk yeast tRNA. Each reaction contains 5 ng of pretRNA^{Trp}.

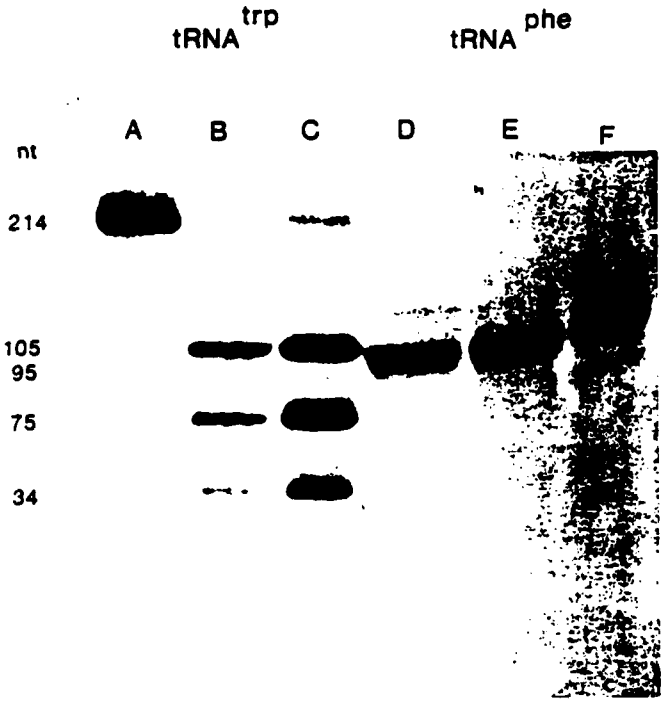
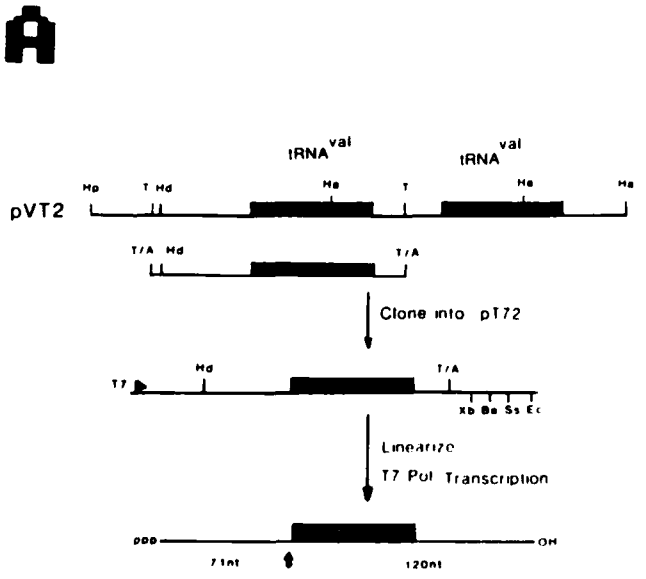


Figure 3. Comparison of halobacterial and yeast pretRNA processing H. volcanii pretRNA^{trp} (lanes A-C) and yeast pretRNA^{phe} (lanes D-F) substrates are processed in the presence and absence of added bulk yeast tRNA. Lanes A and D are no enzyme controls, lanes B and D contain 10 ug of bulk yeast tRNA during the processing reaction and lanes C and F are processing reactions under standard conditions for pretRNA^{trp}. Each reaction contains approximately 5 ng of labeled substrate.



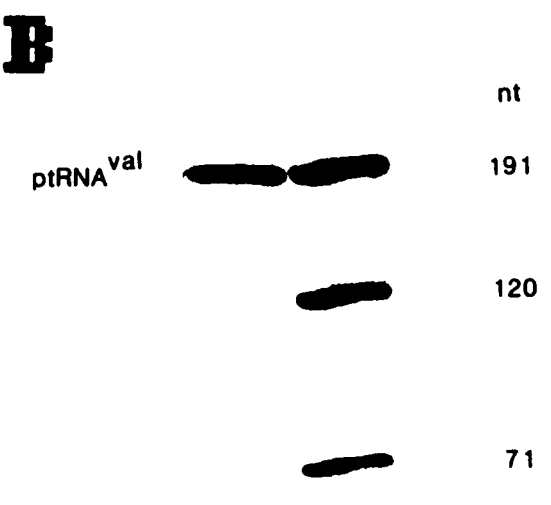


Figure 4. Preparation and RNAase P cleavage of the H. volcanii tRNA^{val} precursor. Panel A, subcloning of the H. volcanii tRNA^{val} gene into the T7 RNA polymerase transcription vector. Panel B, cleavage of the in vitro synthesized pretRNA^{val} substrate by a partially purified H. volcanii RNAase P enzyme.

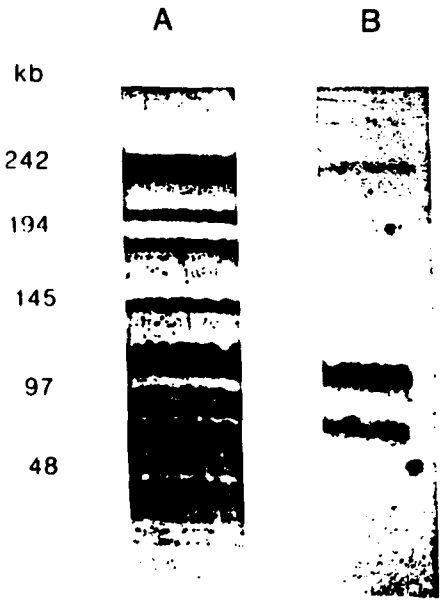


Figure 5. Southern hybridization of pCp 3'- end labeled H. volcanii tRNA to BamHI restriction endonuclease cleaved H. volcanii genomic DNA. Panel A, separation of BamHI restriction fragments of H. volcanii genomic DNA by orthogonal field gel electrophoresis. Panel B, Southern hybridization of separated fragments with 3' end labeled H. volcanii.

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